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**14. ABSTRACT**
The overall goal of this research project is to develop and implement a conceptually innovative strategy for extrinsic regulation of the androgen receptor (AR) to target the transcriptional misregulation mediated by this receptor in prostate cancer. This strategy utilizes bifunctional molecules that simultaneously interact with AR and with components of the chromatin remodeling machinery. In the second year of funding, a paper outlining the early stages of the study was accepted for publication. Additionally, thorough characterization of HDAC inhibitor-based structures revealed that localizing HDACs to promoters via AR leads is not efficacious. However, targeting an epigenetic reader, Brd4, lead to robust and reliable extrinsic control of full-length nuclear receptor function. In the final year of funding, this latter model will be fully extended in the prostate cancer system.

**15. SUBJECT TERMS**
androgen receptor, HDAC inhibitor, Brd4, JQ1, prostate cancer

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# INTRODUCTION

Androgen receptor (AR) is an allosterically regulated transcription factor that binds to both the endogenous steroids testosterone and 5α-dihydrotestosterone and to a range of synthetic ligands. AR is central to prostate cancer pathogenesis and its reactivation is a hallmark of castration-resistant prostate cancer (CRPC), an aggressive and terminal illness for which there is no effective treatment. Despite this disease progression, most androgen-refractory prostate cancers continue to rely on AR for their survival; thus it remains an important therapeutic target.[1] Historically, approaches to modulate AR have focused on targeting the ligand-binding pocket with small molecules that sculpt the surface of the receptor in unique ways.[2] This indirect remodeling of the receptor binding surface results in the recruitment of different native binding partners. Although a powerful strategy, it has already been found that mutation of either binding surface (i.e. AR or cofactor) can occur, leading to, for example, antagonists that later become agonists.[Error! Bookmark not defined.,1] Even with the development of second-generation anti-androgens and small molecules that target sites other than the ligand-binding domain of AR, new small molecules are urgently needed that can suppress AR function and do not rely on allostery to elicit their effect.[3,4,5,6] In this project, we are developing an innovative alternative strategy to specifically steer and extend the repertoire of receptor-coregulator partnerships. By targeting ligand and substrate pockets in the receptor and coregulator complexes, we propose to bridge the two using novel small molecule bifunctional recruiters (Figure 1).

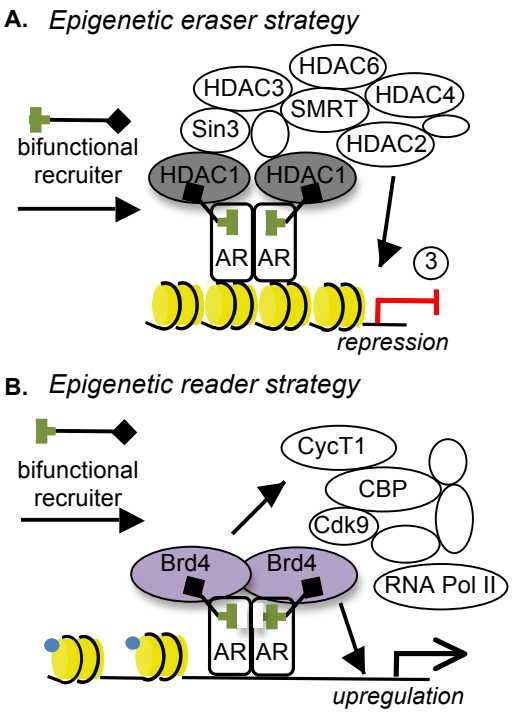



**Figure 1**. **A.** Epigenetic eraser strategy to block AR function. Bifunctional recruiters contain a high affinity AR ligand and an isoform-selective HDAC inhibitor (HDACi) can recruiting corepressor complexes to AR. One functional consequence of this targeted recruitment could be deacetylation of chromatin, repressing transcription. **B.** Epigenetic reader strategy to enhance AR function. Bi-functional recruiters contain a high affinity bromodomain inhibitor to recruit Brd4 to AR regulated promoters. One functional consequence of this mode of targeted recruitment could be up-regulation, as depicted. However, transcriptional inhibition may be observed due to steric blockade at some genes.

Our original experimental plan focused upon one of the best-characterized mechanisms of transcriptional inhibition, the recruitment of large corepressor complexes that harbor histone deacetylase (HDAC) activity (Figure 1A).[Error! Bookmark not defined.,7,8,9,10,11, 12] By appropriately linking HDAC inhibitors to high-affinity nuclear receptor ligands, we proposed to generate a new class of molecules that recruit transcriptionally repressing complexes to AR, a predicted consequence being the suppression of AR genomic function. As outlined in more detail in the body of this progress report, the targeted bi-functional molecules performed in vitro as designed and in cellular model systems; the preliminary studies supporting our model were recently accepted for publication (Appendix I). However, in cellular models of prostate cancer, no significant gene-specific or phenotypic effects were observed. In this project period (months 13-24) we have thus implemented the alternative strategy outlined in the original proposed work plan and illustrated in Figure 1B. In this strategy, we use the potent bromodomain inhibitor JQ1 to recruit Brd4 and thus extrinsically alter the transcriptional status of the targeted genes. **Importantly, this strategy has been successfully implemented in a full-length nuclear receptor mode in this funding period**. We are thus poised to assess the JQ1-based molecules in genome-wide expression analyses to define the pattern of up- and down-regulated genes in prostate cancer. Additionally, the phenotypic effects of AR-bromodomain dimerization will be measured in prostate cancer models in the final funding period.

## BODY

*Summary of **Task 1** goals:* The primary focus of Task 1 was the design and synthesis of bi-functional molecules consisting of high-affinity AR ligands linked to class-selective HDACi. Full in vitro characterization of the bi-functional molecules comprises this Task. Molecules that demonstrated affinity for both targets comparable to unmodified inhibitors (within 2- to 4-fold) were carried on to *Task 2*.

*Accomplishments of **Task 1**, Months 13-24:*

As outlined in the Year One progress report, all goals of Task 1 were accomplished in the first twelve months of the Project with the exception of the synthesis of PD106 conjugates of andarine. This was completed and assessment of those molecules carried out in Task 2. A manuscript including preliminary results from Task 1 was recently accepted for publication and can be found in Appendix I (Jonas W. Højfeldt, Osvaldo Cruz-Rodríguez, Yasuhiro Imaeda, Aaron R. Van Dyke, James P. Carolan, Anna K. Mapp and Jorge A. Iñiguez-Lluhi, *Molecular Endocrinology* **2013** accepted for publication).

*Summary of **Task 2** goals:* Bifunctional molecules developed in the previous task will be examined in different cell lines (PC-3, LNCaP, VCaP) for their ability to modulate exogenous (luciferase) and endogenous (PSA, ERG) AR-driven reporters. Control experiments will also be performed to compare the effects of bifunctional molecules and HDACi alone (i.e. HDACi without an AR-targeting moiety). **(Months 4-18)**. Bifunctional recruiters that demonstrate cellular activity will be further examined as outlined in *Task 3*. If bi-functional molecules do not show efficacy in these assays, replacement of the HDACi recruitment moiety with an alternative inhibitor of chromatin modifying activities will be examined.

*Accomplishments of **Task 2**, Months 13-24*

<u>Three-hybrid assay to assess recruitment by bi-functional molecules</u>

The data from Task 1 established that the bi-functional ligand design attenuated the affinity of each component ligand to its respective targets by only 2-5-fold. In this set of experiments, the goal was to assess if this would translate to recruitment of both targets to a promoter through a three-hybrid assay. In this experimental setup, a fusion of the HDAC of interest (HDAC3 is shown) to VP16 is expressed in the cells. As shown in Figure 2, if the bi-functional molecule interacts with AR and with HDAC3 then the potent VP16 activation domain should lead to transcriptional activation of a luciferase reporter gene or an endogenous gene.

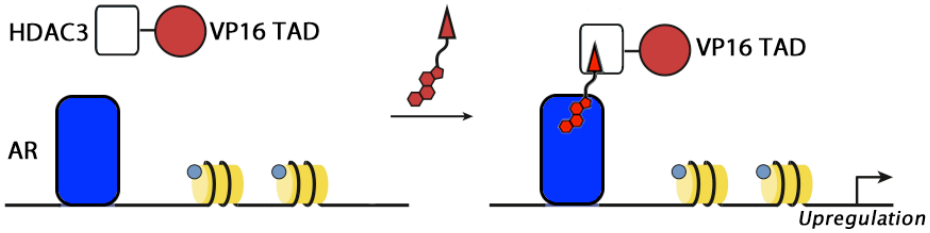



**Figure 2. Schematic of 3-hybrid assay used to test bi-functional molecules.** In this assay the the HDAC of interest is fused to the potent transcriptional activator VP16; as an example, an HDAC3-VP16 construct is illustrated. Thus, if the bifunctional molecule interacts with both AR and the targeted HDAC at the promoter, then transcriptional up-regulation will be observed.

*Andarine-O5-PD106 and hAR ± VP16-HDAC3*

HEK293T cells were transfected with plasmids encoding full-length androgen receptor (1 µg), a luciferase reporter containing the prostate specific antigen promoter (1 µg), a fusion protein of HDAC3 with the VP16 TAD at its N-terminus and a FLAG tag at the C-terminus (1 µg), and a CMV driven β-galactosidase reporter (40 ng) using Lipofectamine 2000 (10 µL) in Opti-MEM media. Additionally, a second aliquot of cells were transfected using the same cocktail replacing HDAC3 with pBSSK+ as a negative control. The

cells were allowed to recover overnight following transfection and were then trypsinized and plated on a 96 well plate (20,000 cells per well). After adhering for eight hours, cells were dosed with various concentrations of Andarine, Andarine-O5-$N_3$, Andarine-O5-PD106, or DMSO as a vehicle control for eighteen hours. The cells were then lysed and the luciferase and β-galactosidase activity were assayed. Activation of the PSA reporter plasmid was normalized using β-galactosidase activity and is reported as fold activation over DMSO.

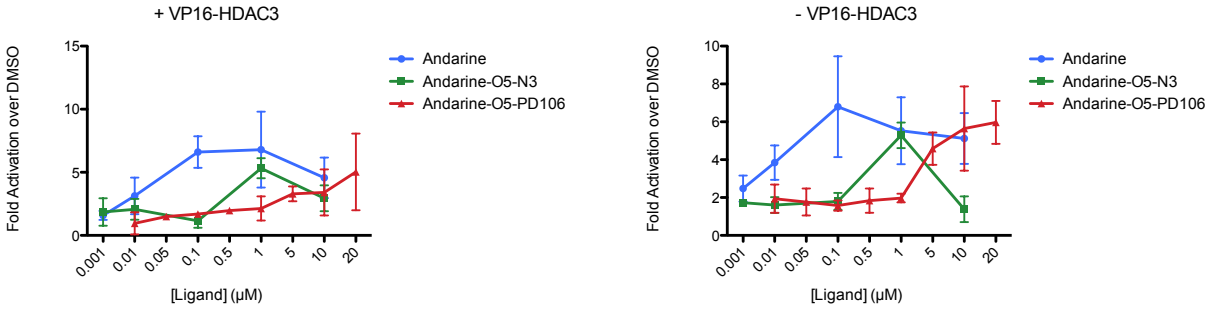



**Figure 3**. **Results from 3-hybrid experiments with HDAC3-VP16 fusion protein.** Data shown is the average of three independent experiments with the indicated errors (SDOM).

The moderate activation of the reporter plasmid by andarine is consistent with data reported in the literature and confirms that the cells were effectively transfected.[14] Andarine-O5-PD106 is also capable of activating the reporter to a moderate extent, though only at high concentrations and without any dependence on the presence of the VP16 fusion. Thus, the activation observed for the bifunctional molecule is not the result of dimerization of the target proteins, but instead is likely due to the inherent attenuated activation potential of the andarine component of the molecule.

*Andarine-O5-PD106 and hAR + HDAC1-VP16*

HEK293T cells were transfected as described above with the exception that the plasmid encoding the HDAC3 fusion protein was replaced with a plasmid encoding an HDAC1 fusion protein with the VP16 TAD at its C-terminus. The cells were allowed to recover and dosed as described above before luciferase and β-galactosidase activity were assayed.

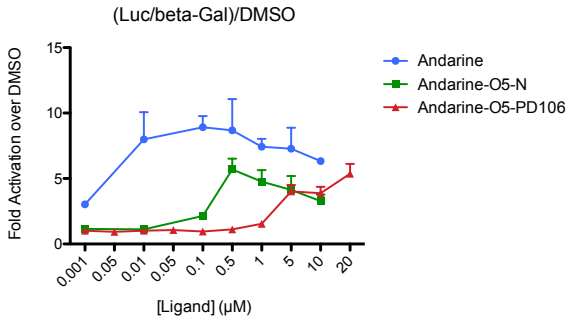


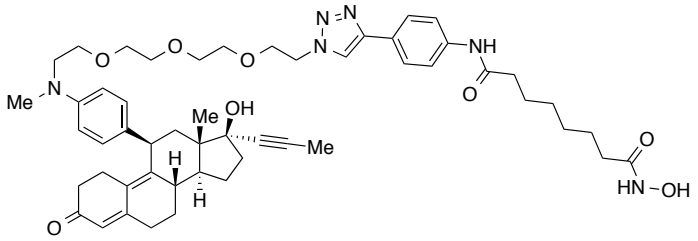

**Figure 4**. **Results from 3-hybrid experiments with HDAC1-VP16 fusion protein.** Data shown is the average of three independent experiments with the indicated errors (SDOM).

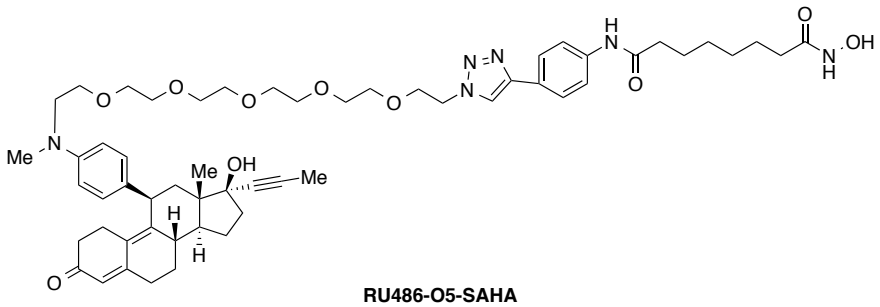
PD106 is a slow, tight-binding Class I (HDAC1, HDAC2, HDAC3, and HDAC8) selective HDAC inhibitor.[15] After the observation that Andarine-O5-PD106 could not dimerize AR and HDAC3, the

experiment was repeated using an alternative Class I HDAC, HDAC1. Dosing with andarine again resulted in moderate activation of the reporter, confirming the cells were effectively transfected. Consistent with the results using VP16-HDAC3, the bifunctional molecule failed to dimerize the target proteins and the observed activation at high concentrations of the conjugate is again likely due to the inherent activation potential of the andarine component of the molecule.

*RU486-O3/O5-SAHA and hAR ± VP16-HDAC3*

After the disappointing results of the previous experiments we hypothesized that the use of an anti-androgen as the AR targeting agent would lead to an AR form that would be more easily controlled extrinsically. Towards that end, we designed and synthesized two bifuunctional molecules composed of the antiantrogen RU486 conjugated to the pan-specific HDAC inhibitor SAHA through a polyethylene glycol linker.[7,16] Two molecules containing different linker lengths were prepared and assessed to determine the impact of linker length on the activity of the molecules. HEK293T cells were transfected as described for the previously described VP16-HDAC3 three-hybrid experiment. Following the transfection, cells were allowed to recover and were then dosed with various concentrations of RU486-O3-SAHA, RU486-O5-SAHA, RU486-O3-$N_3$, andarine, or DMSO as a vehicle control for eighteen hours before luciferase and β-galactosidase activity were assayed.



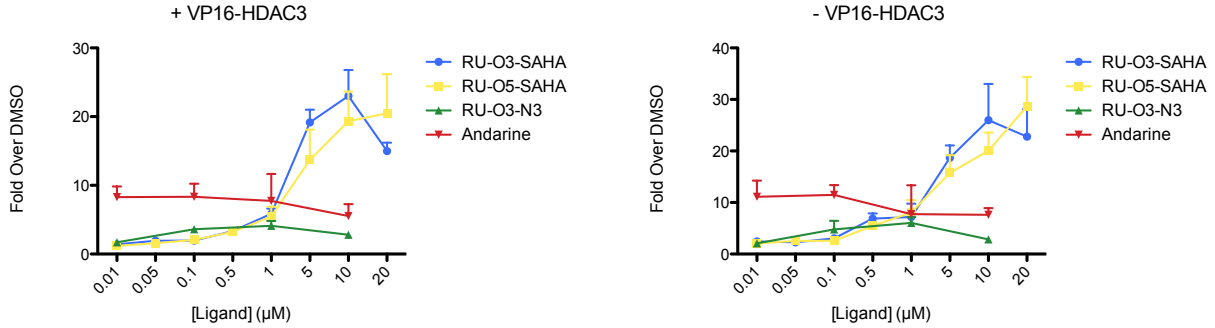
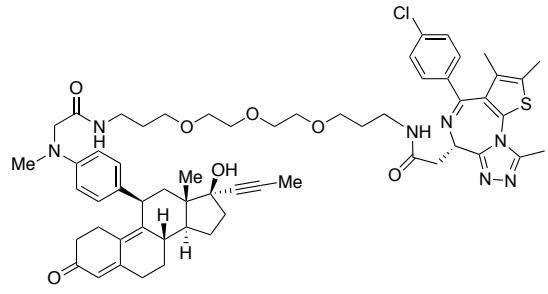




**Figure 5. Three-hybrid assessment of RU486-based bifunctional molecules**. Above are the structures of the two RU486-based molecules, differing only in the linker length connecting the two functional units. Results are the average of three experiments, with the indicated error (SDOM).

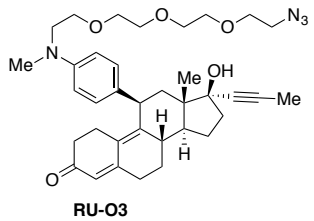
Consistent with previous experiments, andarine led to moderate activation of the reporter, confirming the effective transfection of the cells. Dosing with RU486-O3-$N_3$ led to very low activation (~1-4 fold over DMSO), consistent with the function of the parent molecule as a partial agonist antiandrogen.[16] The two conjugates tested led to significant activation at high concentrations of small molecule, with no apparent dependence on linker length. Interestingly, this activation is also not dependent upon the presence of the VP16 containing HDAC3 fusion protein. These data suggest that the conjugate is not dimerizing the target proteins with the desired effect, as transcription is significantly potentiated rather than abrogated even in the absence of the VP16 fusion protein.

Several reports suggest that treatment with low concentrations of SAHA can lead to enhanced transcriptional output in exogenous reporter systems.[17-19] The loss of transcriptional activity at high doses of the *trans* addition (> 5 μM; data not shown) is consistent with the reported $IC_{50}$ values for SAHA in cellular proliferation assays against several prostate cancer cell lines.[20] Thus, the activity of the bifunctional molecule is explained by the activities of the individual component molecules, RU486 and SAHA. Furthermore, the conjugate displays lower potency and efficacy than the addition of the molecules *in trans*, which is likely a result of the diminished binding affinity of the proteins for the molecules due to the modifications made in order to link them.

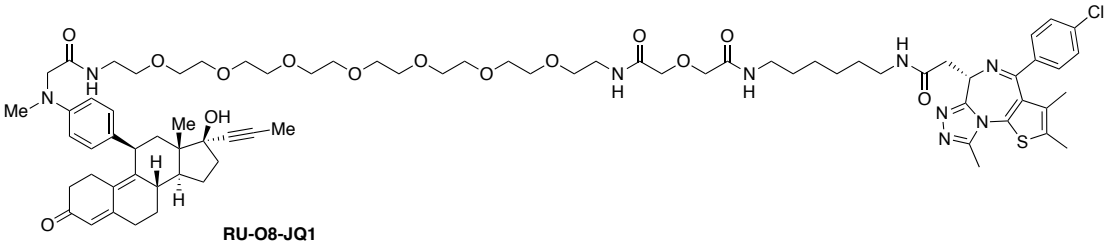
Re-design of bi-functional molecule Our data from Task 1 and results from our model system (Appendix 1) illustrate the fundamental feasibility of extrinsic control of androgen receptor function through bi-functional recruiters. The primary difficulty with the HDACi-based molecules appears to be ineffective recruitment at the promoter level. Thus we sought to replace the HDACi moiety with an alternative recruiter and target epigenetic reader proteins (i.e. bromodomains) instead of epigenetic erasers (i.e. HDACs). (S)-JQ1 is a highly potent and selective inhibitor of bromodomain 4 (BRD4) which is known to intereact with critical components of the transcriptional machinery (e.g. CDK9, cyclin T1, RNA Pol II). Because our bifunctional molecules are constructed modularly, it is straightforward to conjugate existing advanced intermediates with JQ1 using our synthetic strategies developed for Task 1



**RU-O3-JQ1**



**RU-O3**



**RU-O8-JQ1**

*Functional assessment of JQ1-based agents* In contrast to the AR three hybrid system used to assess the

HDACi-based conjugates, to test these constructs we have targeted full length human glucocorticoid receptor (hGR) as we and others have observed a more robust dynamic range in the transcriptional response. The schematic for his assay is shown below in Figure 6. We examined a range of promoters regulated by GR to test the effect of promoter architecture on recruitment and functional outcome. Although one would expect the primary response of Brd4 recruitment to be transcriptional up-regulation, in some cases steric effects may lead to repression.

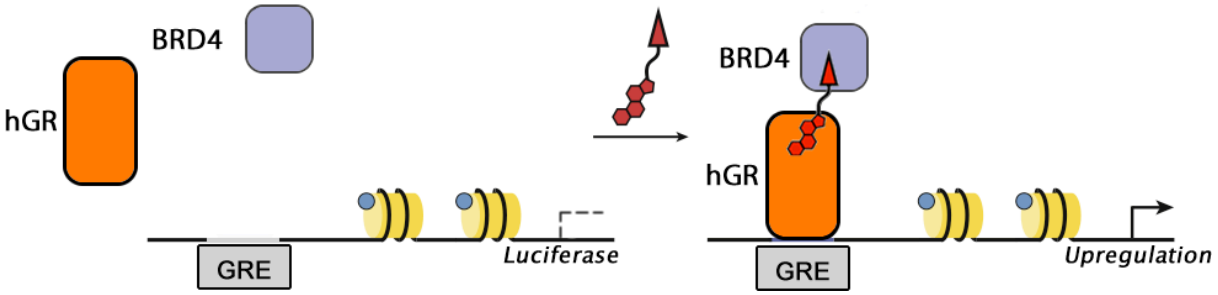



**Figure 6. Functional assay for testing JQ1-based bifunctional molecules.** In this assay, bifunctional molecules are tested for there ability to localize endogenous human glucocorticoid receptor (hGR) to a reporter vector and upregulated the transcription of luciferase. Upstream of the luciferase gene is a glucocorticoid response element (GRE) sequence for hGR binding based on a consensus GRE or a GRE found in an endogenous, hGR-regulated gene. In this assay format, bifunctional molecule recruitment of BRD4 yields higher levels of activation relative to a glucocorticoid antagonist in both a dose-dependent and time-dependent manner.

Shown in Figure 7 are the results of examining four distinct GR-regulated promoters. Briefly, U2-OS cells were seeded in a six-well plate and transfected with a blank pCDNA3 vector (750 ng) along with plasmids encoding full length human glucocorticoid receptor (250 ng), β-galactosidase (500 ng), and firefly luciferase (500 ng) using Lipofectamine 2000 (5 uL) in serum-less media. A consensus glucocorticoid response element (GRE) or GRE based on a glucocorticoid receptor gene was cloned upstream of the luciferase expression gene. Following overnight recovery, cells were trypsinized and replated in charcoal-stripped media in a 96-well plate at a seeding density of 5,000 cells per well. After adhering for six hours, cells were dosed with RU-O3-N3, RU-O3-JQ1, or DMSO as a vehicle control for eighteen hours, or the specified time. The cells were then lysed and assayed for luciferase or β-galactosidase activity. Luciferase activity was normalized to β-galactosidase activity and is reported as fold activation over DMSO. As expected, the pattern of transcriptional response varied significantly with promoter architecture. The FKBP and Pal promoters, for example, exhibited a robust response relative to RU-486 bearing only the linker. Although not shown explicitly in these graphs, trans addition did not produce these effects, indicating that the physical linkage between RU-486 and JQ1 is a requirement. Additionally, a construct bearing the enantiomer of JQ1, a molecule that cannot bind to Brd4, has no activity in these assays. Finally, increasing the linker length between JQ1 and RU-486 did not lead to enhanced activity. Thus, we continue to focus on the RU-O3-JQ1 conjugate.

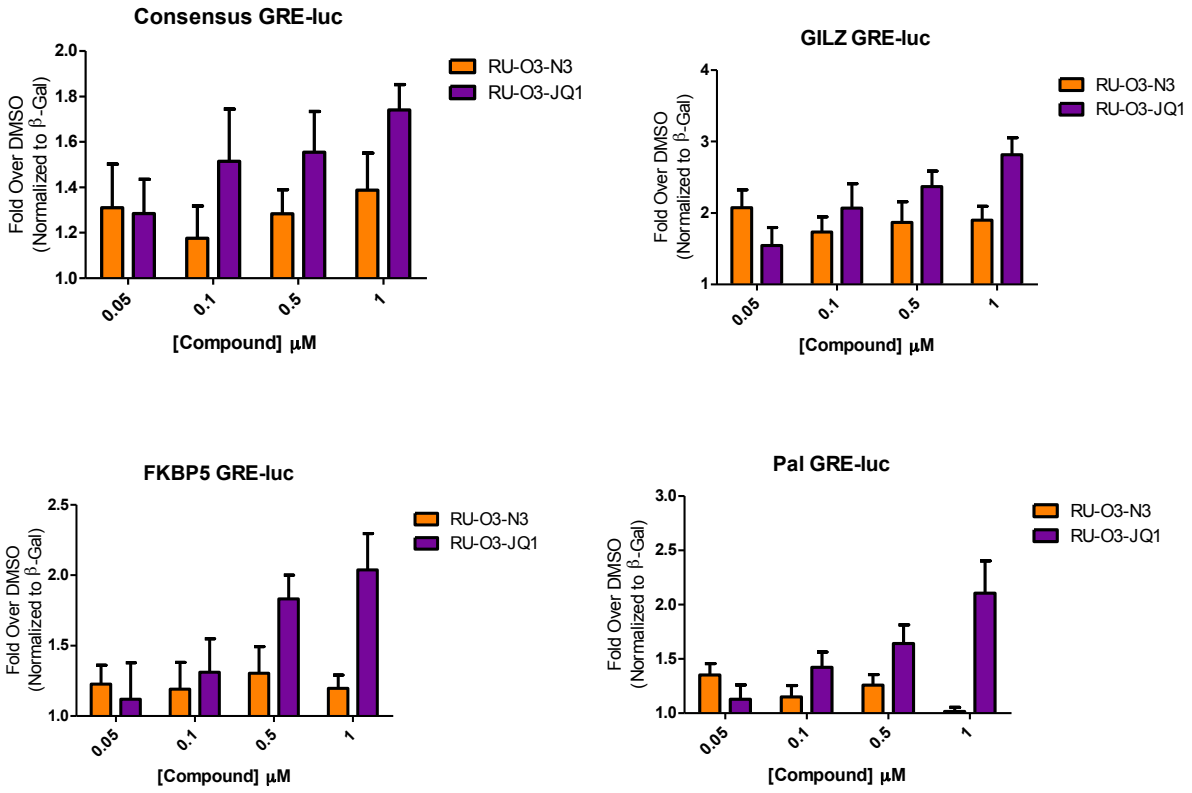



**Figure 7. Results from assays with full length human GR as the nuclear receptor.**

In addition to the dose-response observed in the data of Figure 7, we observed a time-dependence (Figure 8). In the case of the consensus GRE and GILZ promoters, for example, significant additional activity was observed after 8 hours.

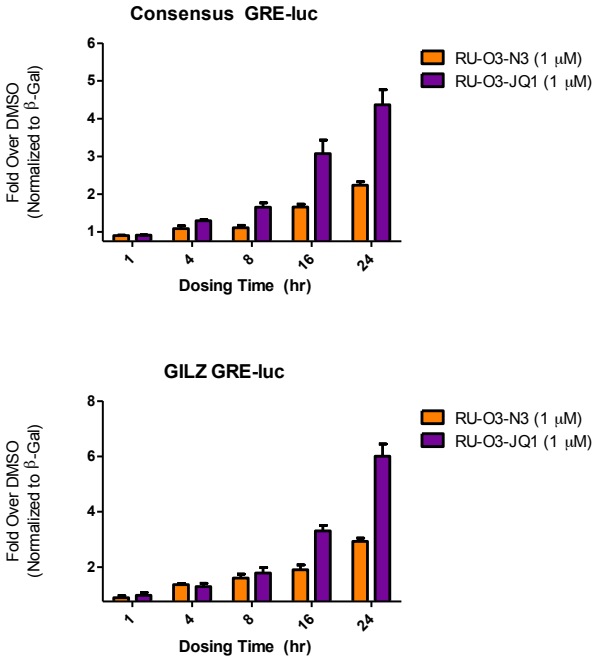



**Figure 8.** Time course data for GR-dependent promoters

Taken together, these exciting data indicate that bifunctional recruiters that target epigenetic readers such as

JQ1 have robust in a full length nuclear receptor. These molecules will also serve as ligands for AR so we are well-poised to test this in our prostate cancer model system.

*Assessment and future milestones for **Task 2**:* As outlined in the Task 2 summary our current efforts are focused on functional characterization of the bifunctional molecules based upon the JQ1 scaffold. This will be accomplished through the examination of endogenous AR-regulated genes (PSA, ERG for example) in addition to luciferase assays. In addition to the RU486 molecules, we will examine andarine-based and bicalutamide-based constructs synthesized and evaluated as previously outlined. The best molecules will be carried on to Task 3.

*Summary of **Task 3** goals* The goals of Task 3 as originally articulated focused on assessing the changes in acetylation pattern, cell growth and apoptosis stimulated by the HDACi-based inhibitors; in other words, these experiments were designed to test the endogenous functional consequences of treating cells with the HDACi-based bifunctional molecules. Although the design of the bifunctional molecules has been modified to incorporate bromodomain inhibitors such as JQ1, the new experimental goals remain aligned: we will examine both cell growth and apoptosis in LnCAP cells upon treatment with the RU486-JQ1 conjugates. Additionally, as andarine and bicalutamide conjugates emerge from the Task 2 experiments, they will similarly be assessed. Further, we will characterize the most promising molecules by genome-wide expression analysis. These latter experiments are particularly important as they will enable assessment of which genes are up-regulated by Brd4 recruitment, which are repressed and how this varies based upon the identity of the AR ligand. These experiments are the top priority for the third year of funding as they will be the most informative for selecting molecules for Task 4 assessment.

## KEY RESEARCH ACCOMPLISHMENTS

• Evaluated first and second generation suite of HDACi-based bifunctional models in cellular models of AR function in an extensive suite of experiments. These data established that while dual targeting is feasible in cell-free environments, in the context of full-length receptor at endogenous genes, intrinsic pathways dominate.

• Optimal linker identity and length was identified and validated in a model study for both binding and function. These results were accepted for publication (see Appendix 1)

• A re-design of the bifunctional molecules targeted the epigenetic reader Brd4 as the recruitment moiety. The previously designed synthetic strategy enabled rapid preparation and assessment of this scaffold.

• The JQ1-based conjugates can be used, for the first time, to extrinsically regulate the function of a full-length receptor at endogenous promoters. This is a critical step forward.

## REPORTABLE OUTCOMES

• A model study that enabled identification of key elements of a *functioning* bifunctional transcriptional regulator has been accepted for publication (see Appendix 1)

• The Task 1 and Task 2 data have been presented by (former) postdoctoral fellow Dr. Aaron Van Dyke at the 2013 Bioorganic Chemistry Gordon Research Conference and at the 2013 ACS Northeast Regional Meeting. Graduate students JP Carolan and Steve Sturlis have presented this work at the 2013 Novartis Symposium at the University of Michigan (see Appendix 2 for abstracts)

## CONCLUSIONS

The overall goal of this research project is to develop and implement a conceptually innovative strategy for

down-regulating androgen receptor: the creation of bifunctional molecules that simultaneously bind to the androgen receptor and to chromatin modifying complexes. In this way, AR function can be extrinsically controlled. In this second year of funding, we made critical progress towards this goal with the demonstration that targeting an epigenetic reader, Brd4, is a successful strategy. In addition, the workflow is well established such that additional molecules are being streamlined through the process. In the next funding period, the focus will be on full functional characterization of the molecules in more advanced prostate cancer model systems.

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# APPENDICES

# APPENDIX 1

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You are being carbon copied ("cc:'d") on an e-mail "To" "Jorge A. Iñiguez-Lluhí" iniguez@umich.edu
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10 Dec 2013

Dr. Jorge Iñiguez-Lluhí
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Dear Dr. Iñiguez-Lluhí and co-authors,

We are pleased to inform you that your revised manuscript, "Bifunctional Ligands Allow Deliberate Extrinsic Reprogramming of the Glucocorticoid Receptor", manuscript number ME-13-1343R1, has been accepted for publication in Molecular Endocrinology.

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# Bifunctional Ligands Allow Deliberate Extrinsic Reprogramming of the Glucocorticoid Receptor

--Manuscript Draft--

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| **Abstract:** | Therapies based on conventional nuclear receptor ligands are extremely powerful yet their broad and long term use is often hindered by undesired side effects which are often part of the receptor's biological function. Selective control of nuclear receptors such as the glucocorticoid receptor using conventional ligands has proven particularly challenging. Because they act solely in an allosteric manner, conventional ligands are constrained to act via cofactors that can intrinsically partner with the receptor. Furthermore, effective means to rationally encode a bias for specific coregulators are generally lacking. Using the glucocorticoid receptor as a framework, we demonstrate here a versatile approach based on bifunctional ligands that extends the regulatory repertoire of the glucocorticoid receptor in a deliberate and controlled manner. By linking the macrolide FK506 to a conventional agonist (Dexamethasone) or antagonist (RU-486), we demonstrate that it is possible to bridge the intact receptor to either positively or negatively acting coregulatory proteins bearing an FK506 binding protein domain. Using this strategy, we show that extrinsic recruitment of a strong activation function can enhance the efficacy of the full agonist dexamethasone and reverse the antagonist character of RU-486 at an endogenous locus. Notably, extrinsic recruitment of HDAC1 reduces the ability of GR to activate transcription from a canonical GR response element while preserving ligand-mediated repression of NFkB. By providing novel ways for the receptor to engage specific coregulators, this unique ligand design approach has the potential to yield both novel tools for GR study and more selective therapeutics. |

# Bifunctional Ligands Allow Deliberate Extrinsic Reprogramming of the Glucocorticoid Receptor

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**Abbreviated title:** *Bifunctional Ligand Control of Nuclear Receptors*

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**Key words:** Nuclear receptors, glucocorticoid receptor, transcriptional regulation, ligand binding, transcription coregulators, bifunctional ligands, dissociated glucocorticoid, ligand design.

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**Disclosure statement:** The authors have nothing to disclose

## Abstract

Therapies based on conventional nuclear receptor ligands are extremely powerful yet their broad and long term use is often hindered by undesired side effects which are often part of the receptor's biological function. Selective control of nuclear receptors such as the glucocorticoid receptor using conventional ligands has proven particularly challenging. Because they act solely in an allosteric manner, conventional ligands are constrained to act via cofactors that can intrinsically partner with the receptor. Furthermore, effective means to rationally encode a bias for specific coregulators are generally lacking. Using the glucocorticoid receptor as a framework, we demonstrate here a versatile approach, based on bifunctional ligands, that extends the regulatory repertoire of the glucocorticoid receptor in a deliberate and controlled manner. By linking the macrolide FK506 to a conventional agonist (Dexamethasone) or antagonist (RU-486), we demonstrate that it is possible to bridge the intact receptor to either positively or negatively acting coregulatory proteins bearing an FK506 binding protein domain. Using this strategy, we show that extrinsic recruitment of a strong activation function can enhance the efficacy of the full agonist dexamethasone and reverse the antagonist character of RU-486 at an endogenous locus. Notably, extrinsic recruitment of HDAC1 reduces the ability of GR to activate transcription from a canonical GR response element while preserving ligand-mediated repression of NFkB. By providing novel ways for the receptor to engage specific coregulators, this unique ligand design approach has the potential to yield both novel tools for GR study and more selective therapeutics.


## Introduction

Synthetic glucocorticoids are one of the most widely used pharmacologic agents mainly because of their potent anti-inflammatory and immunosuppressive effects. Given that maladaptive inflammation or inappropriate immune responses are a central part of many chronic diseases, glucocorticoids are an invaluable therapeutic tool in a wide range of conditions including arthritis, asthma, lupus and allergy and are an important element of immunosuppressive regimens for organ transplantation (1). Despite these

well- established and sometimes life-saving therapeutic applications, conventional glucocorticoid therapy is severely limited due to undesirable side effects. These are mainly due to the profound metabolic changes in energy and protein metabolism that endogenous glucocorticoids set in motion as an adaptive response to transient stress. Consequently, pharmacologic glucocorticoid excess leads to hyperglycemia, visceral adiposity, and insulin resistance as well as muscle wasting and osteoporosis. Pharmacologic approaches that mitigate the metabolic effects of glucocorticoids while preserving their immunomodulatory activity would be a major therapeutic advance. This however, has proven elusive despite intense efforts (2-4).

The effects of glucocorticoids are mediated by the glucocorticoid receptor (GR) a prototypic member of the nuclear receptor superfamily of sequence-specific ligand regulated transcription factors. Upon binding of an agonist to the C terminal ligand binding domain (LBD), GR translocates to the nucleus and localizes to specific loci through a central zinc finger region capable of direct recognition of specific sequences or through tethering to other transcription factors. From these sites, GR influences the transcription of target genes by nucleating the assembly of specific coregulatory complexes through protein-protein interactions (5). The receptor orchestrates this process by integrating multiple signals (6), including variations in the target DNA sequence (7), intracellular signaling cascades, post-translational modifications (8, 9) and uniquely, small cell-permeable ligands that bind to the LBD (Fig.1A).

The canonical mode of action of endogenous ligands involves their binding to the LBD and consequent reorientation of helix 12 leading to the engagement of the C-terminal activation domain (AF2). In concert with additional activation functions in the N-terminal region, these conformational changes alter the interaction surfaces for transcriptional coactivators and corepressors that are responsible for controlling chromatin remodeling as well as transcriptional initiation and elongation (10).

Glucocorticoid responses are the integration of complex patterns of tissue-specific gene expression and involve both activation and repression of target genes (11, 12). Limiting metabolic side effects while

preserving immunomodulatory actions therefore would require a clear identification of the "on pathway" desirable responses as well as those involved in undesirable side effects and importantly, effective means to elicit one without the other. The ability of glucocorticoids to repress expression of multiple proinflammatory cytokines is a central component of its anti-inflammatory effects whereas the induction of metabolic enzymes such as PEPCK is an important component of the metabolic response. Initial efforts have therefore focused towards dissociated agonists that can support repression while minimizing transactivation but have met with limited success (2-4).

The difficulties associated with conventional NR ligands are likely to be due in large part to the fact that they act solely through allosteric modulation of receptor conformation and are therefore constrained to recruit from a closed set of cofactors that are within the intrinsic interaction envelope of the receptor. This puts a limit to the range of achievable functional effects. Furthermore, despite the incorporation of novel protein dynamics criteria (13), it is not yet possible to rationally design into a conventional ligand the ability to recruit specific coregulator complexes or to generate a particular pattern of gene expression. A further challenge is that coactivators involved in transactivation such as GRIP1 also participate in transcriptional repression mechanisms (14, 15). Furthermore, the development of clinical resistance is a significant problem, particularly in cancer therapy (16).

In a significant departure, we describe here a novel strategy that circumvents the limitations of conventional allosteric ligands and frees the receptor to engage in novel extrinsic functional interactions that are open to rational design. The approach leverages the versatility of GR bifunctional ligands which although have proven useful in three hybrid assays (17), have never been exploited to regulate the intact GR in its native context. By linking prototypic agonist and antagonist GR ligands to the small molecule FK506, we have thus generated bifunctional ligands that can bind the intact receptor and bridge it to designed transcriptional activators or corepressors containing the FK506 binding protein (FKBP) domain (Fig.1B). Using this strategy, we demonstrate for the first time that the regulatory repertoire of the native receptor at endogenous genomic loci can be expanded by directing the deliberate recruitment of extrinsic

coregulators. This strategy allows the designed reprogramming of the intact receptor and can dramatically enhance or completely reverse the efficacy of conventional ligands. By eliciting unique responses, this new class of GR ligands can thus serve as mechanistic probes to discern which are the relevant "on pathway" transcriptional responses required for specific therapeutic effects and which are not required or causative of undesired side effects (18). Notably, this work demonstrates the viability of exploiting druggable small molecule binding sites in coregulator complexes for extrinsic recruitment to the receptor and opens the way for the purposeful recruitment of endogenous transcriptional coactivators and repressors to identify and drive therapeutically desirable responses.

# Materials and Methods

### Synthesis and binding affinity of GR bifunctional ligands

The macrolide FK506 was installed via a polyethylene glycol linker to a Dexamethasone (Dex) derivative where the 21-hydroxyl group is replaced by a thioether linkage (19). For the RU486 derivatives, a similar linker strategy was employed using the aniline group of RU486 as the attachment point. Parallel compounds lacking the FK506 moiety were prepared as controls. Detailed information on compound synthesis is included as *supplemental data*. Binding affinities for Dex, RU486 and their derivatives were determined experimentally by radiolabeled competition binding assays using an extract from Hi5 insect cells expressing an N-terminally His and GFP tagged rat GR. For extract generation, Hi5 cells were infected with H10 eGFP TEV GR baculovirus at a MOI of 4 and lysed 48 h post infection with a Dounce homogenizer in 20 mM Hepes pH 7.4, 1 mM EDTA, 5 % glycerol, 20 mM sodium molybdate, 5 mM DTT and complete® (Roche) protease inhibitor cocktail (1tablet/10ml). The homogenate was centrifuged at 60,000 RPM at 4°C for 30 min in a TI-35 rotor (Beckman). The clear supernatant was aliquoted, flash frozen and stored at -80°C until use. Binding assays were carried out in 10 mM Hepes pH 7.4, 1 mM EDTA, 20 mM sodium molybdate buffer in a 96 well plate format. The receptor extract (30 μg/well) was incubated with a mixture consisting of 10 nM 1,2,4,6,7–[$^3$H] Dexamethasone (Perkin

Elmer) and increasing concentrations of test ligand at 4°C in a final volume of 50 µl. After a 2 h incubation, 100 µl of a dextran coated charcoal solution (1% charcoal, 0.2% dextran in 10 mM Hepes pH 7.4, 1 mM EDTA) was added to each well, followed by centrifugation for 2 min at 1000×g. Aliquots (80 µl) of the resulting supernatants were transferred to Optiplate 96 well plates (Perkin Elmer), supplemented with 120 µl of MicroScint 40 (Perkin Elmer) and read on a TopCount NXT microplate scintillation counter. Data were fit to a single binding site competition model using GraphPad Prism version 5.0 (GraphPad Software, La Jolla California USA).

**Plasmids, cell culture and transfections**

Plasmids p6R GR (20) and pRSV β-gal (21) are Rous sarcoma virus promoter-driven expression vectors for WT rat GR and β-galactosidase respectively. pGBR 6.1 is a luciferase based reporter harboring a 500 bp intronic region of the human FKBP5 gene and has been described previously (11, 22). The 5× NFκB Luciferase reporter (23) was a kind gift of Dr. Gabriel Nuñez, (University of Michigan). Plasmids pLIC FKBP and pNLS-FKBP are CMV promoter driven expression vectors for a protein bearing the SV40 NLS followed by human FKBP1A. The VP16 activation domain sequence (residues 411-456) was inserted between the NLS and FKBP1A sequences of pNLS-FKBP to generate pNLS-VP16-FKBP. A similar strategy was used to insert the mouse HDAC1 sequence at the same position of pLIC FKBP. For all constructed plasmids, relevant regions were sequenced and are available upon request. Human embryonic kidney (HEK 293T) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, (Life Technologies, Carlsbad, CA, USA). For functional assays, $3\times10^6$ cells seeded in 100 mm plates were transfected 24 h later with 50 ng of p6R GR, 400 ng pGBR 6.1, 200 ng pCMV β-gal and 200 ng of either pNLS-FKBP, pNLS-VP16-FKBP or pLIC HDAC1-FKBP using Lipofectamine (Life Technologies) according to the manufacturer's instructions. Cells were trypsinized 16 h post-transfection and resuspended in DMEM supplemented with 10% heat inactivated charcoal stripped FBS and seeded onto 96 well plates at a density of $2\times10^4$ cells per well. After an additional 8 h, cells were treated with either vehicle or the indicated ligands and harvested 16 h later.

Cells transfected with the 5× NFκB Luciferase reporter plasmid were treated with 10 ng/ml of hTNFα (Sigma) in addition to the indicated ligands. Luciferase and β-galactosidase activities were determined as described previously (20). For endogenous gene expression analysis, cells ($1.5\times10^4$/well) were seeded onto 24-well plates and transfected 16 h later with 50 ng of p6RGR and 25 ng of either pLIC FKBP, pNLS VP16 FKBP or pLIC HDAC1-FKBP using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Sixteen hours post-transfection, cells were treated for an additional 6 h with either vehicle or the indicated compounds. Total RNA was isolated using RNeasy RNA isolation kits (Qiagen) and 500 ng of each RNA sample was used to synthesize cDNA using iScript cDNA synthesis kits (Bio-Rad). Quantitative real-time PCR (qRT-PCR) reactions were carried out in duplicate in a Roche 480 LightCycler using QuantiTect SybrGreen reagents (Qiagen) and primers for human RPL19 (Forward, 5'-ATGTATCACAGCCTGTACCTG-3'; Reverse 5'-TTCTTGGTCTCTTCCT-CCTTG-3') and S100P (Forward, 5'-CGGAACTAGAGACAGCCATGGGCAT-3'; Reverse 5'-AGA-CGTGATTGCAGCCACGAACAC-3') genes. LinRegPCR (Ver 11.0) (24) software was used to estimate S100P mRNA levels relative to the reference RPL19 transcript. For protein level analysis, parallel cultures were harvested directly in SDS PAGE sample buffer. After brief sonication, lysates were centrifuged for 2 min at 16,000×g and supernatants were resolved by SDS PAGE and transferred to PVDF membranes (Millipore). FKBP12 (AbCam ab58072) and BuGR2 antibodies were used at 1:2000 dilution. Images were captured in a Li-Cor Odyssey® Fc reader using stabilized goat anti-mouse HRP conjugated antibodies (Pierce) and Super Signal West Femto Chemiluminescence reagents (Pierce).

# Results

### Design of bifunctional ligands

Using the glucocorticoid receptor as a paradigm and building on the significant experience in structure-activity relationship for GR ligand conjugates (17, 19, 25-28), we created bifunctional molecules based on the agonist Dex and the antagonist RU486. The least perturbing derivative of Dex

that allows facile conjugation with other molecules has been termed SDex, in which the 21-hydroxyl group is replaced by thioether linkages (19) (Fig. 2). RU486 has been linked to bile acids through its aniline group and retained antagonistic activity (28). Using a polyethylene-glycol linker as a spacer, we have thus conjugated these ligand derivatives to the natural product FK506 (Fig. 2). Our choice was based on the extensive experience using FK506 conjugates for ligand induced protein complex assembly (29, 30). Compounds bearing a linker only were synthesized as controls (Fig. 2).

The binding affinity of the conjugates for full-length GR was determined in a radiolabeled Dex competition binding assay. As can be seen in Fig. 3A, the affinities of SDex-$O_3$-OMe and SDex-$O_2$-FK506 are approximately 50 and 100 nM. Although this is 10 and 20 fold lower than the affinity of Dex (5 nM), these values are in a range comparable to the affinity of endogenous steroids such as cortisol (20 nM). Although conjugation of FK506 to SDex, lowers the binding affinity a modest 2-fold, this difference is not due to effects of binding cellular FK506-binding proteins since binding studies done in the presence of free FK506 yielded similar results (*not shown*). In the case of the RU486 derivatives, both compounds displayed comparable affinities (~90 nM), which are less than an order of magnitude (~6-fold) lower than that of unmodified RU486 (Fig. 3B). From this analysis, it is clear that the synthetic strategy yielded bifunctional ligands that retain significantly high affinity for GR.

**Extrinsic recruitment of a designed coactivator enhances GR ligand efficacy**

FK506-binding proteins such as human FKBP1A bind with sub-nanomolar affinity to FK506 and the fact that this high-affinity interaction is retained even when the macrolide is conjugated to other molecules has made this pairing the basis for multiple successful small-molecule mediated protein recruitment strategies (31). As a first approach, we designed and constructed a coactivator fusion protein consisting of a nuclear localization signal, the strong transcriptional activation domain of the herpes simplex virion protein 16 (VP16), and FKBP1A and examined its ability to modulate GR activity by monitoring the transcriptional output of a GR-stimulated reporter driven by a natural GR enhancer

sequence. As can be seen in Fig. 4A, in the absence of any fusion protein, the Dex-derived bifunctional molecules (SDex-$O_2$-FK506 and SDex-$O_3$-OMe) led to a dose-dependent enhancement of activity with both compounds achieving maximal responses comparable to that of the parent agonist Dex. As expected from their somewhat reduced binding affinity, these compounds activated with lower potency relative to Dex. Interestingly, even though SDex-$O_2$-FK506 has discernably lower affinity than SDex-$O_3$-OMe, both compounds activated with comparable potencies suggesting an advantage for the FK506 conjugate. From this data, it is apparent that despite the presence of the linker and FK506 moiety, the bifunctional ligands remain cell permeable and retain full efficacy revealing a significant degree of steric tolerance by the native receptor.

In notable contrast, in the presence of the VP16-FKBP fusion (Fig. 4B), the FK506-conjugated ligand (SDex-$O_2$-FK506) displayed a nearly two fold increase in maximal activity relative to Dex and the corresponding $EC_{50}$ of 0.76 nM reflects a ~7 fold increase in potency relative to the linker only ligand (SDex-$O_3$-OMe). Notably, these effects were specific to the FK506 conjugated ligand since the presence of the fusion did not appreciably alter the response to Dex or SDex-$O_3$-OMe. Thus, the enhancement depends on both the fusion and the FK506 moiety of the ligand. These data argue that the enhanced activation in the presence of the fusion is due to extrinsic recruitment by the bifunctional ligand. If this is indeed the case, it can be anticipated that the effect should be disrupted by excess unconjugated FK506 (32). In support of this prediction, increasing concentrations of free FK506 reduced the activity of the bifunctional ligand (Fig. 4C) until it was essentially indistinguishable from the behavior of the ligand lacking FK506 (except for a small increase in basal activity at the highest concentration of FK506). As expected, the dampening effect of free FK506 depended on the presence of the fusion and was not observed for SDex-$O_3$-OMe (*not shown*). Taken together, these results clearly indicate that by using the extrinsic recruitment strategy, bifunctional ligands endow GR with the ability to activate transcription well beyond what can be achieved with one of the most efficacious conventional ligands. This strategy

opens the way for uniquely high efficacy ligands that could serve to overcome clinical resistance or to restore function due to inborn deficits in GR transactivation.

**Extrinsic recruitment of HDAC1 selectively reduces agonist efficacy in activation but not repression contexts**

Therapeutically, GR ligands that have reduced transactivation efficacy but retain full agonism in repression contexts have been sought after since they may display more favorable efficacy vs. side effect profiles. Since these properties have proven to be difficult to obtain using conventional ligands, we sought to build upon our initial results and use the extrinsic control approach to explicitly design and implement this desired regulatory outcome. Since recruitment of corepressor complexes is a common strategy employed by transcription factors to negatively regulate transcription, we constructed a designed coregulator where FKBP1A is fused to the histone deacetylase HDAC1, an enzyme which is an integral component of multiple corepressor complexes (33, 34). In the presence of this coregulator, we then examined the ability of SDex based bifunctional ligands to mediate GR activity in both a canonical activation context (as examined in the experiments above, Fig. 5A *left*) as well as in a repression context (Fig.5A *right*) where GR inhibits TNFα-stimulated NFκB activity. As in the case of pro-inflammatory cytokine genes, GR is recruited to DNA indirectly by tethering to NFκB and inhibits transcription in an agonist-dependent manner, likely at a step downstream of RNA pol II recruitment (35, 36). As can be seen in Fig. 5B *left*, in the presence of the FKBP-HDAC1 coregulator, the maximal response elicited at the activation context by SDex-$O_2$-FK506 was significantly blunted, reaching only ~30% of the response induced by Dex. In contrast, the fusion did not alter the activity elicited by either Dex itself or SDex-$O_3$-OMe (compare to Fig. 4). These results indicate that extrinsic recruitment of HDAC1 can successfully oppose the intrinsic efficacy of a conventional ligand in an activation context. In notable contrast, analysis of the tethering repression context (Fig 5B, *right*) revealed that both the control and FK506 conjugated SDex bifunctional ligands supported GR-mediated inhibition of NFκB to the same extent as Dex (Fig. 5B). Since this same repression activity profile was observed in the absence of any fusion

protein (*data not shown*), this indicates that the FKBP-HDAC1 coregulator did not interfere with the ability of the ligands to repress in this context. As expected given their lower intrinsic affinity, the bifunctional ligands were less potent than Dex in both the activation and repression contexts. Taken together, the results indicate the successful establishment of the desired outcome and underscore the deliberate design potential of the extrinsic recruitment approach.

**Efficacy switch from antagonist to agonist through extrinsic recruitment**

The above experiments show that in an activation context, it is possible to positively or negatively modulate the efficacy of an agonist ligand by the judicious extrinsic recruitment of coregulators. To probe the design versatility and scope of this approach, we sought to determine to what extent the regulatory effects of extrinsically recruited factors can be dissociated from the efficacy intrinsic to the GR binding moiety of the bifunctional ligand. To this end, we examined the properties of RU486 based ligands where the receptor binding moiety is an antagonist. As can be seen in the left panels of Fig. 6, in the absence of any fusion, RU486 and its derivatives showed no detectable activation of the GR responsive promoter (Fig. 6A, left). The compounds, however, are active and cell permeable since they are able to antagonize the activity of 3 nM Dex in a dose-dependent manner (Fig. 6B, left). Interestingly, even though both RU486 based ligands have indistinguishable affinities (Fig. 3B), the FK506 conjugate antagonized Dex with an $IC_{50}$ ~6 fold lower than the linker only ligand. This makes the FK506 conjugate comparable to the parental RU486, despite its lower binding affinity. This indicates that the FK506 moiety imparts additional properties to the ligand which could include effects on cellular accumulation of the ligand.

In contrast to the above data, in the presence of the VP16-FKBP fusion, RU486-$O_3$-FK506 became an effective inducer of transcriptional activation reaching a maximal activity nearly as strong as that of Dex (Fig. 6A, *right*). Interestingly, the response is biphasic since activation is reduced at the highest concentration. This behavior however, is consistent with the properties of bifunctional ligands, since at

sufficiently high concentrations, formation of binary ligand-protein complexes is favored over the ternary one (32, 37). It is also notable that half-maximal activation by the FK506 conjugate occurred at concentrations ($EC_{50}$ ~3nM) significantly lower than the intrinsic receptor affinity (~90 nM). Importantly, and similarly to the case of the Dex derivatives, the activity depends on both the fusion protein and the FK506 moiety and can be disrupted with free FK506 (Fig. 6A and *not shown*).

Consistent with a large gain in efficacy, the behavior of the ligands in the presence of 3nM Dex (Fig. 6B *right*) indicated that RU486-$O_3$-FK506 behaved as an agonist and increased activity beyond 3 nM Dex. The fact that the peak activity is higher than that observed with RU486-$O_3$-FK506 alone also suggests that at these concentrations, where mixed occupancy is likely, cooperation between intrinsic (Dex bound GR) and extrinsic (RU486-$O_3$-FK506 bound GR) mechanisms is occurring. As anticipated from competitive displacement, the behavior at the highest concentrations is comparable to that of RU486-$O_3$-FK506 alone. Furthermore, the presence of the VP16-FKBP fusion did not appreciably alter the antagonistic behavior of the parental RU486 or the conjugate lacking FK506 indicating that the observed effects require both the appropriate coregulator and the FK506 moiety in the bifunctional ligand. Taken together, these data clearly show that the regulatory effects elicited through extrinsic recruitment can be fully dissociated from the intrinsic properties of the GR binding moiety. The versatility of the approach is such that it allows for the predictable reprogramming of the transcriptional output of GR such that the behavior of a ligand can be completely reversed from an antagonist to an agonist.

**Regulation in an intact chromatin environment through extrinsic recruitment**

The implementation of the overall GR transcriptional program *in vivo* occurs in the context of a complex chromatin environment and any successful ligand strategy must be able to operate under these circumstances. To demonstrate that GR-extrinsic transcriptional control can also be achieved at endogenous GR target genes in their native chromatin context, we focused on the S100P gene. The basal expression of this gene in HEK293T cells is comparatively low and can be stimulated ~50 to 100 fold by

Dex only upon GR expression. These properties indicate that the receptor is a major determinant of S100P transcription and make it a suitable target for analysis. In cells co-expressing FKBP alone, both of the SDex derivatives at 100 nM (which is comparable to their dissociation constant) were able to activate the S100P gene ~ 20 fold (Fig. 7A, *left*). In contrast, in cells co-expressing the VP16-FKBP fusion, S100P expression was three fold higher in the presence of SDex-$O_2$-FK506 compared to the control ligand SDex-$O_3$-OMe, which lacks the FK506 moiety (Fig.7A *center*). In fact, the activity elicited by the FK506 conjugate reached levels comparable to those obtained with the same concentration of Dex (which in comparison, corresponds to a 20 fold excess over its own dissociation constant). On the other hand, in cells expressing the HDAC1-FKBP fusion, S100P expression in response to SDex-$O_2$-FK506 was reduced by half in comparison to SDex-$O_3$-OMe (Fig.7A, *right*). Thus, for both the positive and negative modulation, successful extrinsic control can be demonstrated in a manner that depends on both the appropriate coregulator partner as well as on the FK506 moiety in the ligand. A parallel analysis of the effects of the RU486 based ligands (Fig.7B) revealed that in the presence of FKBP alone, the ligands did not appreciably activate S100P expression. This is expected from the intrinsic antagonist nature of RU486. In the presence of the VP16-FKBP fusion however, the FK506 conjugate successfully activated S100P expression more than 10 fold compared to the ligand lacking the FK506 moiety. Importantly, western blot analysis demonstrated successful expression of the fusion proteins (Fig. 7C, *bottom*) and comparable expression of the receptor (Fig. 7C, *top*). Taken together, the data clearly indicate that the extrinsic recruitment approach can be readily used to reprogram the expression of genes in their natural context and thus is amenable to further development for potential therapeutic applications.

## Discussion

The ligand design we have implemented demonstrates that it is possible to independently manipulate intrinsic as well as extrinsic pathways of GR control and that their combinatorial coupling via bifunctional ligands can yield a variety of regulatory outcomes in both activation and repression contexts (Table 1). The approach is instructive in multiple ways since it reveals mechanistic features of GR

function and opens up numerous design opportunities for its directed manipulation. The ability of the intact GR LBD to accommodate both agonist and antagonist based bifunctional ligands is notable since the predicted exit trajectory of the linker from the LBD based on structural information is quite different. For the Dex derivatives, the linker attachment site is very close to the surface and projects outward between helix 3 and 11 on the opposite side of helix 12. The linker is likely accommodated with minor movements of nearby residues such as T739 and Ile 747. The ability of the bifunctional ligands on their own to mount maximal responses comparable to Dex argues that the linker is accommodated while preserving a functional AF2. For the RU406 derivatives, the linker extends from the aniline moiety, which is responsible for preventing helix 12 from adopting an active conformation and is already solvent exposed. Despite opposite exit points from the LBD and a relatively short linker, both bifunctional ligands are proficient for the recruitment of designed coregulators, which indicates a significant degree of flexibility and steric tolerance. These properties are favorable to the further development of the extrinsic recruitment approach.

The ability of the HDAC1-FKBP coregulator to limit transactivation by GR is also revealing. On the one hand, the inhibitory effect does not appear to be due to steric hindrance because FKBP alone or fusions to other proteins of comparable size are inactive (not shown). Furthermore, although our data is consistent with the established role of HDAC1 as a component of corepressor complexes, recent data based on the functional effects of HDAC1 knockdown has been interpreted as HDAC1 playing a positive role in GR transactivation (38). Whether this reflects indirect effects of HDAC1, or more complex interactions as has been suggested recently (39), remains to be determined. It is also important to note that HDACs can play key scaffolding roles in corepressor complexes that do not depend on their HDAC activity (34, 40). Consistent with this view, initial data indicates that the catalytic activity of HDAC1 is not required for its ability to suppress GR transactivation in the extrinsic recruitment approach (not shown). . This would indicate that the catalytic site of HDAC1 could be targeted by bifunctional ligands to recruit functional corepressor complexes. Importantly, targeting HDAC1 allowed selective reduction

of GR transactivation while preserving agonist mediated repression. Such an outcome is difficult to obtain with conventional ligands because the same AF-2 directed coregulators, such as GRIP1 can participate in both contexts (15).

The ligand design we have implemented has unique properties that make it amenable to directed design strategies. The nearly independent manipulation of the properties of both the receptor binding moiety and the additional chemical functionality is a significant advantage. This modularity extends the receptor's own design where the DNA and ligand specificity have divergently evolved to generate distinct receptors with unique properties and functions. The substantial level of flexibility afforded by this approach greatly increases the types of ligands that can be envisioned. Although we have demonstrated a controlled change in efficacy with prototypic agonist and antagonist receptor ligands, the strategy could be combined with compounds with some conventional dissociated properties (2) or the arylpyrazole nonsteroidal series (3) that display some cell and gene selective properties to leverage both intrinsic and extrinsic effects. The experiments described here demonstrate that the added functionality afforded by the bifunctional ligand can be used in explicit design efforts to direct a desired transcriptional output and override the intrinsic properties of the receptor binding moiety. This also means that ligands with very high affinity but with weak efficacy could be used as scaffolds. Such a strategy could counteract the mild penalty in affinity incurred by the introduction of the linker.

It is also important to note that in addition to affecting the pharmacodynamic properties of a receptor ligand, the linker as well as the additional chemical functionality in the bifunctional ligand can influence its pharmacokinetics and this can be advantageous. Thus, studies of an analogous Dex conjugate series indicate that the thiourea linker used here confers favorable properties both for cellular permeability (44) and trans-scleral transport in the context of ocular delivery (45). Our data also indicates that the FK506 moiety enhances the cellular potency of Dex (Fig. 4) as well as RU486 based bifunctional ligands (Fig. 6) relative to their intrinsic receptor affinities (Fig. 3), an effect observed even in the absence of designed FKBP fusion proteins (Fig 6B, *left*). The enhanced potency may be a reflection of increased cellular

uptake or retention provided by the FK506 group, particularly since FK506 can serve as a substrate and inhibitor of drug efflux transporters such as MDR1 (41). Notably, the favorable pharmacokinetic properties provided by FK506 have been recently demonstrated for drug conjugates both *in vitro* and *in vivo* (42, 43). Similarly, conjugation of GR antagonists to bile acids has been explored as a means to target GR antagonism to the liver (28). The oral bioavailability, tissue distribution and microsomal stability of such conjugates (28) indicate that GR bifunctional ligands can have pharmacokinetic properties suitable for further clinical development.

The experiments presented here depend on genetic manipulation of the designed coregulator. Although this may be incorporated as part of gene-therapy strategies, transition to bifunctional ligands acting on purely endogenous proteins will obviously depend on appropriate functionalities that can target coregulator complexes. In this regard, the recent progress by our group (46-48) and others (49) in the development of small molecules that can mimic activation domains offer some clear opportunities (50). Enhancing the agonist efficacy of glucocorticoid ligands in this manner could provide a means to overcome or alleviate steroid resistance, which is an important clinical problem in diseases such as asthma (51, 52), nephrotic syndrome (53) and malignancies such as acute lymphoblastic leukemia (54). Similarly, such ligands could restore function to carriers of mutations in GR that impair interaction with coactivators (55, 56). Indeed, the modular design of our bifunctional ligands argues for the successful incorporation of these chemical motifs to target endogenous regulatory complexes.

Bifunctional ligands made as FK506 conjugates as in this study have intriguing prospects in their own right as mechanistic tools. They can be used to directly recruit specific FKBP-coactivator or corepressor fusions. This can provide a means to not only identify factors that can overcome gene-specific rate-limiting barriers to activation or to provide novel mechanisms of repression for specific GR target genes. For example, extrinsic recruitment of factors implicated in GR mediated repression at tethering sites such as NELF (14) could enhance the repressive effects of agonists in a gene selective manner. By examining multiple cofactors in parallel, this approach could also be used to establish epistatic relationships between

1 them and the gene subsets affected by them. It is precisely this type of knowledge that is required to
2 identify the most desirable regulatory profile for a given therapeutic application. Furthermore, the
3 approach could also be used to establish or monitor specific epigenetic marks at GR targeted loci in a
4 ligand dependent manner as has been recently illustrated for Oct4 (57). Clearly, the strategy outlined here
5 has significant potential and opens the possibility of instructive ligand design not only for GR but for the
6 entire nuclear receptor class.

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## Footnotes

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[3]Abbreviations used: NR, Nuclear Receptor; LBD; Ligand binding domain; GR, Glucocorticoid receptor; Dex, Dexamethasone; FKBP, FK506 binding protein.

# Table

**Table 1.** *Outcomes of combinatorial intrinsic and extrinsic pathways*.

| Pathway | | Maximal effect (relative to Dex) | |
|---|---|---|---|
| Intrinsic | Extrinsic | Context | |
| Ligand character | Cofactor recruitment | Activation | Repression |
| Agonist (Dex) | - | 100 % | 100 % |
| | VP-16 | 200 % | n. t. |
| | HDAC 1 | 30 % | 100 % |
| Antagonist (RU-486) | - | 0 % | n. t. |
| | VP-16 | 75 % | n. t. |

n. t. Not tested

## Figure legends

**Figure 1.** *Extrinsic control of nuclear receptors.* A) Transcriptional regulation by conventional ligands involves binding to the nuclear receptor (NR) and subsequent nucleation of coregulator complexes. The spectrum of targeted complexes is dictated mainly by the intrinsic conformation of the LBD induced by the ligand. B) Bifunctional ligands with unique targeting functionalities may allow the selective recruitment of coregulator complexes not accessible to conventional ligands.

**Figure 2.** *GR bifunctional ligands.* Structures of GR bifunctional ligands based on the agonist dexamethasone (top) or the antagonist RU486 (bottom). Receptor binding, linker and FK506 moieties are indicated.

**Figure 3.** *Bifunctional ligands bind to the glucocorticoid receptor.* Competition binding assays based on displacement of $^{3}$[H] Dexamethasone (10 nM) from GR were carried out as described in *Experimental Procedures.* All curves are fits to a competitive single binding site model. Values for the calculated dissociation constant ($K_d$) are indicated in the inset. A) Competition using Dexamethasone (Dex) or SDex derived ligands. B) Data for RU486 based ligands.

**Figure 4.** *Directed enhancement of efficacy using bifunctional GR ligands.* Dose-response curves for transcriptional activation by Dex, SDex-$O_3$-OMe and SDex-$O_2$-FK506 in HEK 293T cells expressing A) GR alone, or B) co-expressing GR and the fusion protein FKBP-VP16. Note the bifunctional ligand and fusion-dependent enhancement in efficacy. C) Dose-response curves for SDex-$O_2$-FK506 in the absence (filled squares) or presence of 0.1 (gray triangles) or 1 μM (open triangles) of free FK506 in cells coexpressing GR and the fusion protein FKBP-VP16. Note that at the highest concentration of FK506, the response reverts to that seen in the absence of extrinsic recruitment. Data represent the average ± SEM of at least 3 experiments performed in triplicate and are expressed as a percentage of the activity obtained with 100 nM Dex.

**Figure 5.** *Suppressed transactivation with preserved transrepression using extrinsic recruitment.* A) Diagram depicting the GR mediated activation (left) and repression (right) contexts for functional assays. The bifunctional ligands with associated legends are also shown. B) Dose-response curves for transcriptional activation by Dex, SDex-$O_3$-OMe and SDex-$O_2$-FK506 in HEK 293T cells co-expressing GR and the fusion protein HDAC1-FKBP are shown on the left. See Fig. 4 for comparison. Transcriptional repression of TNFα stimulated NFκB activity by Dex and bifunctional ligands in the presence of the fusion protein HDAC1-FKBP is shown on the right. Data represent the average ± SEM of at least 3 experiments performed in triplicate and are expressed as a percentage of the activity obtained with 100 nM Dex (*left*) or 10 ng/ml TNFα alone (*right*). The basal activity in the absence of TNFα was less than 1%.

**Figure 6.** *Antagonist to agonist conversion through extrinsic recruitment.* A) Dose-response curves for transcriptional activation by RU486 based ligands in HEK 293T cells expressing GR alone (left), or co-expressing GR and the fusion protein FKBP-VP16 (right). B) Effect of the ligands in the presence of 3 nM Dex. Data represent the average ± SEM of at least 3 experiments performed in triplicate and are expressed as a percentage of the activity obtained with 100 nM Dex.

**Figure 7.** *Directed regulation of the endogenous S100P gene by bifunctional ligands.* A) mRNA levels of the S100P gene in response to SDex-$O_3$-OMe and SDex-$O_2$-FK506 in HEK 293T cells coexpressing GR and either FKBP alone (left),VP16-FKBP (center) or HDAC1-FKBP (right). B) Response to RU486 based ligands in cells coexpressing GR and either FKBP alone (gray bars) or the VP16-FKBP fusion (black bars). Data are the averages ± SEM of at least four independent experiments performed in duplicate and are expressed as a percentage of the levels observed in response to 100 nM Dex. Except for

the control FKBP alone data, all comparisons between linker only and FK506 conjugates were statistically significant (two tailed Student's t-test, $p \leq 0.01$). C) Western blot analysis of parallel cultures using anti GR (top panel) or FKBP12 (lower panel). Molecular masses of standards (in kDa) are indicated on the right of each panel and nonspecific species are indicated by asterisks. Predicted molecular masses for the FKBP, VP16-FKBP and HDAC1-FKBP fusion constructs are 15.8, 20.3 and 71.1 kDa respectively. The anomalous migration conferred by the acidic VP16 activation domain has been well described (58).

Figure 1

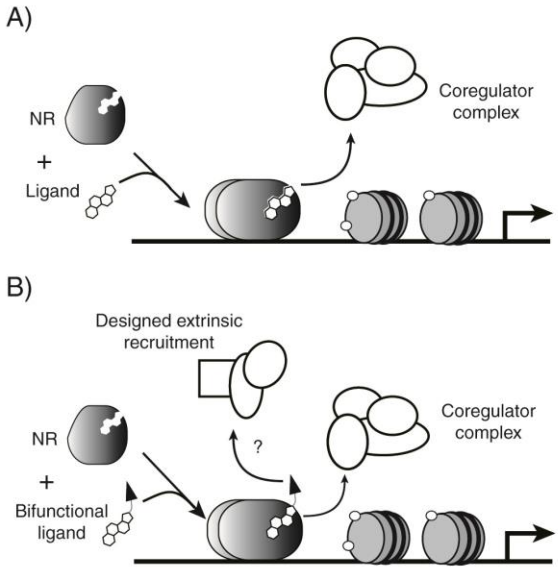


Figure 1

Figure 2

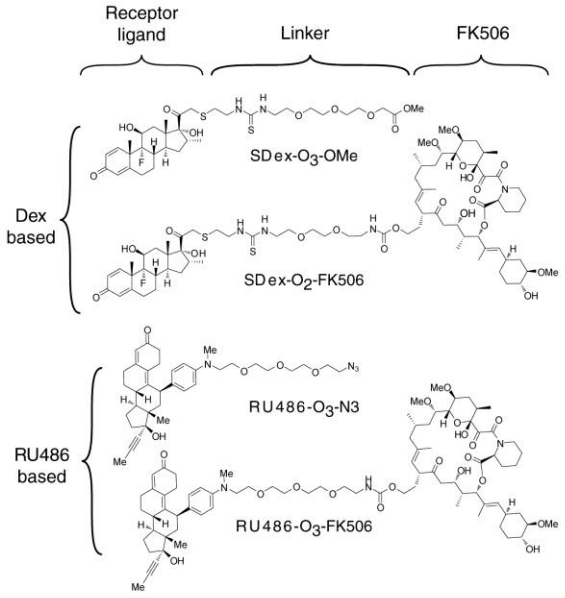


Figure 2

Figure 3

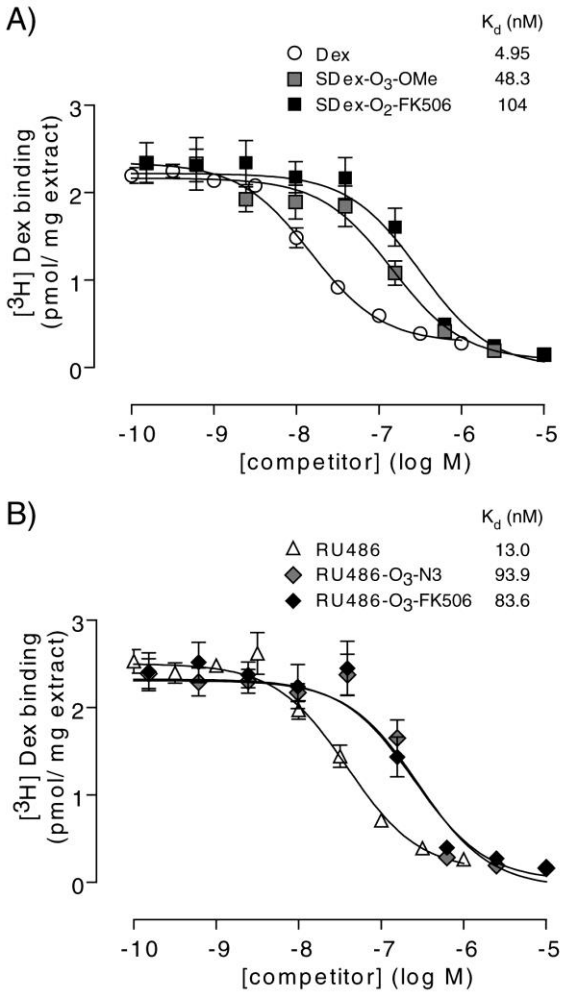


Figure 3

Figure 4

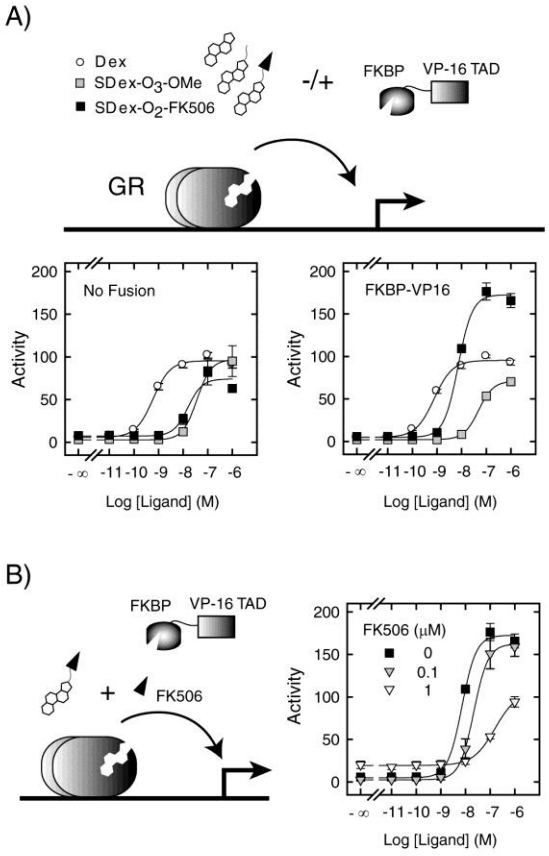


Figure 4

Figure 5

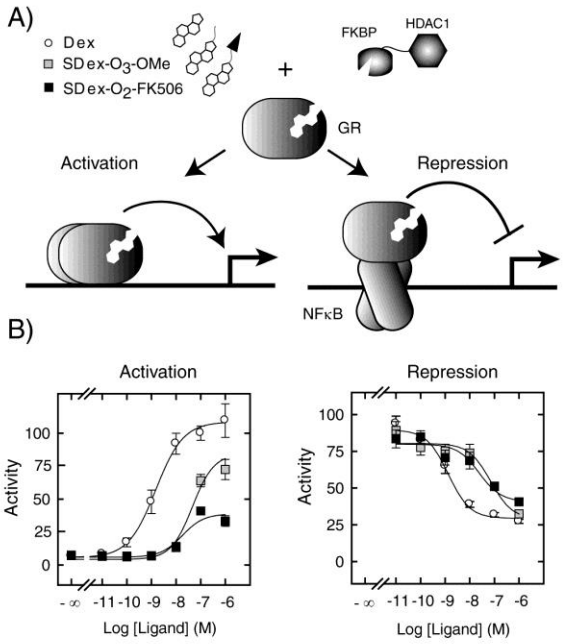


Figure 5

Figure 6

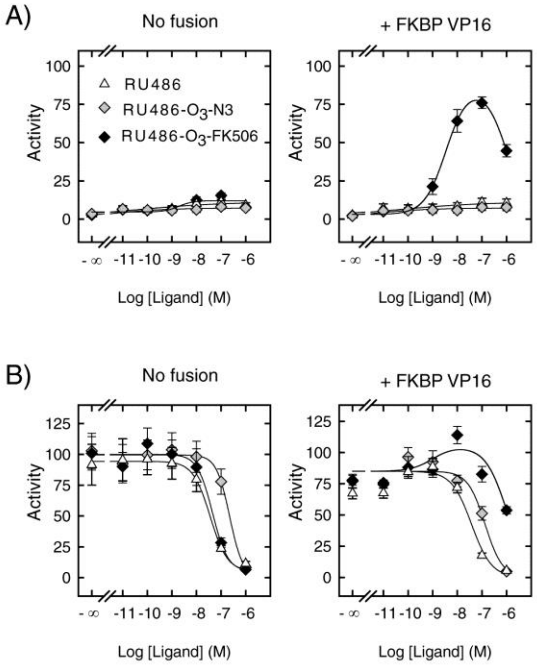


Figure 6

Figure 7

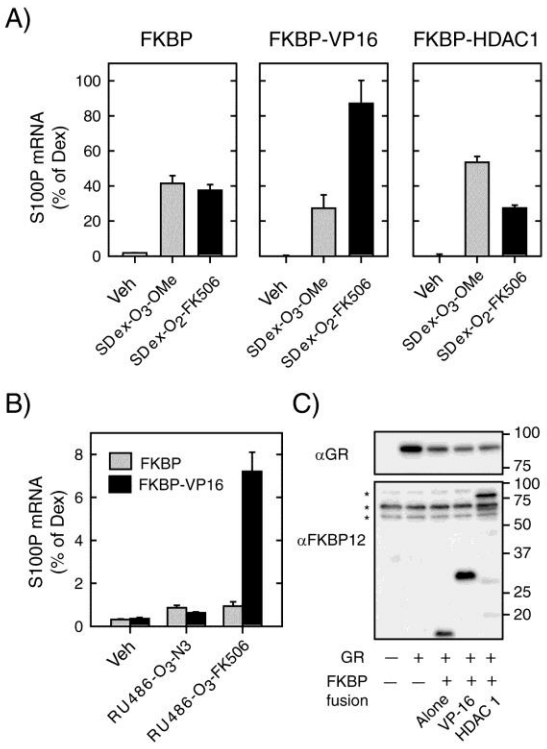


Figure 7

Supplemental Data

Click here to download Supplemental Data: 2013-10-26 GR bifunctional ligands Supplemental data.pdf

# APPENDIX 2: PRESENTATION ABSTRACTS

***Presented by Dr. Aaron van Dyke at the 2013 Bioorganic Gordon Research Conference and the 2013 Regional ACS meeting*** *Dr. van Dyke recently (Sept 2013) assumed an independent position at Fairfield University.*

**Title**: Modulating Gene Expression with Bifunctional Ligands

**Authors**: Aaron Van Dyke, Jun Qi, Jonas Hojfeldt, Osvaldo Cruz, Jorge Iniguez-Lluhi, James Bradner, Anna Mapp

**Abstract**:

Glucocorticoid receptor (GR) is a ligand-inducible transcription factor that regulates gene expression by recruiting protein cofactors to DNA. Errors in this process correlate with a range of human cancers, making GR an important therapeutic target. Historically, GR has been modulated by small molecule ligands that allosterically recruit cofactors. Alternatively, bifunctional ligands that can simultaneously bind GR and cofactor would be powerful tools to directly recruit proteins to DNA. By directly controlling cofactor recruitment to DNA, we aim to achieve greater control over gene expression. As a proof of principle, a collection of bifunctional molecules were synthesized to recruit the artificial activator VP16 to GR. VP16 recruitment activates gene transcription to higher levels than could previously be achieved with classical GR ligands. Current efforts to recruit naturally occurring coactivators in living cells will also be discussed.

***Presented by graduate students Steven Sturlis and James P. Carolan at the 2013 Novartis Symposium***

**Title:** Bifunctional Small Molecules Targeting the Androgen Receptor

**Abstract:**

Aberrant activation of the androgen receptor (AR), a member of the nuclear receptor superfamily, has been strongly implicated in the pathogenesis of prostate cancer, which is the second leading cause of cancer related deaths in men according to the American Cancer Society. The disease is initially treated through androgen ablation therapy to reduce the production of endogenous ligands for the receptor. However, in the majority of cases, the disease progresses to a hormone refractory state, in which AR function is restored, despite low androgen concentrations. At this stage, the disease becomes terminal, underscoring the need to develop novel therapeutic strategies.

One potential strategy to suppress AR regulated gene expression is to recruit corepressor complexes that contain histone deacetylase (HDAC) activity to the promoters of AR controlled genes. HDACs repress gene expression by deacetylating core histones, which results in the tighter compaction of chromatin and reduces the ability of transcription factors to bind. It is hypothesized that the recruitment of

these complexes to AR regulated promoters will lead to the downregulation of genes required for prostate cancer cell survival. Bifunctional molecules capable of binding both AR and HDAC complexes present an attractive approach to implementing this strategy and are currently being investigated.

To this end, a potent AR ligand has been synthesized, to which a polyethylene glycol linker has been appended. Early biological characterization using chromatin immunoprecipitation has demonstrated the ability of the modified ligand to effectively localize and bind AR to DNA. Additionally, an HDAC inhibitor is currently being investigated for potential use within the proposed strategy.